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QUERCETIN DIRECTLY PROMOTES RABBIT OVARIAN STEROIDOGENESIS

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Abstract: The bioflavonoid quercetin is a component of food with numerous biological effects, but its function in reproductive processes remains to be investigated. This study aimed to examine the direct action of quercetin on steroid hormone release in rabbit ovaries. We analysed the effect of quercetin (0, 1, 10, and 100 ng/mL) on cultured rabbit ovarian fragments. The release of progesterone (P4), testosterone (T) and estradiol (E2) were analysed by enzyme immunoassay. Quercetin promoted P4, T, and E2 release by rabbit ovarian fragments. These observations indicate that quercetin can directly stimulate rabbit ovarian steroidogenesis – an essential regulator of reproduction and fecundity. The application of dietary quercetin for control of rabbit reproduction is discussed.

Key Words: nutrition, quercetin, ovary, steroids, rabbits.

INTRODUCTION

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most well-known flavonoids present in plant food with phytoestrogen and antioxidant properties (Moutsatsou, 2007). It is found in high concentrations in fruits and vegetables such as apples, blueberries, cherries, broccoli, grape, leek, lettuce, onion, tomato, citrus fruits and wild herbs (Moutsatsou, 2007; Chen *et al.,* 2010a; Anand *et al.,* 2016). Quercetin is an essential flavonoid in animal nutrition (Santini *et al.,* 2009). It has numerous effects on physiological state and health due to its antioxidant/anti-ageing, anti-inflammatory, antiproliferative, anticarcinogenic and cardioprotective properties (Boots *et al.,* 2008; Chen *et al.,* 2010a; Anand *et al.,* 2016; Sharma *et al.,* 2018). The characteristics and mechanisms of quercetin action in reproduction are insufficiently studied, and studies have shown controversial results. Several authors reported the stimulatory influence of quercetin on ovarian functions: feeding with quercetin reduced ovarian cell apoptosis, promoted proliferation and increased ovarian weight, oocyte quality and litter size in some animals (mice: Shu *et al.,* 2011; Beazley and Nurminskaja, 2016; rabbit: Naseer *et al.,* 2017). Other studies, however, demonstrated the ability of quercetin to suppress reproductive functions. Feeding with quercetin disrupted oestrous cycles, suppressed ovarian folliculogenesis and ovulation, increased ovarian follicular atresia, altered gonadotropin release and decreased litter size in old mice (Shu *et al.,* 2011). Therefore, previous studies have shown inconsistent data regarding the effects of quercetin on female reproductive functions. Furthermore, *in vivo* studies failed to identify the site and mechanisms of quercetin action. The effects of quercetin on mice gonadotropin release (Shu *et al.,* 2011) suggest that it regulates ovarian functions via upstream hormonal regulators. *In vitro* studies demonstrated quercetin uptake by Chinese hamster ovarian (Walgren *et al.,* 2000) and direct quercetin action on ovarian cell steroidogenesis, a key regulator of ovarian folliculogenesis and fecundity (Sirotkin, 2014). Quercetin was able to inhibit aromatase (human: Whitehead *et al.,* 2003; Rice *et al.,* 2006) and progesterone (P4) release (pig: Santini *et al.,* 2009) by cultured ovarian granulosa cells. The mechanisms of its effect and direct influence of quercetin on rabbit ovarian cells have not yet been studied.

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Quercetin could promote rabbit reproduction via changes in steroid hormones –the known regulators of ovarian functions and fecundity (Sirotkin, 2014), but its effects on these hormones in rabbits have not been studied yet.

This study aimed to examine the direct effect of quercetin on steroidogenesis –the release of P4, testosterone (T), estradiol (E2) by cultured rabbit ovarian fragments.

MATERIAL AND METHODS

Isolation and culture of granulosa cells and ovarian follicles

Ovarian fragments were isolated from the ovaries of nulliparous 4-mo-old New Zealand White rabbits and cultured as described previously (Sirotkin *et al.,* 2017). Briefly, the ovaries removed from the does at slaughter were subjected to macro and microscopic inspection to avoid presence of corpora lutea, haemorrhagic and large preovulatory follicles. Two animals with haemorrhagic follicles were excluded from experiments. No ovaries with corpora lutea and preovulatory follicles were found. Thereafter, the ovaries were washed in phosphate buffered saline (PBS) with 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA), placed onto 100-mm diameter culture dishes (Gama, České Budejovice, Czech Republic) and dissected using a blade knife. The resulting 1/8th of the ovaries (approximately 1×1 mm) were weighed and washed thrice in PBS. The ovarian fragments isolated separately from each animal were collected into a separate culture dish. Thereafter, we created the experimental groups, consisting of six ovarian fragments originating from different does. Each group was used to test the effect of quercetin at one dose. The ovarian fragments were cultured in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) 1:1 (BioWhittaker™, Verviers, Belgium) medium supplemented with 10% foetal calf serum (BioWhittaker™) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) in 24-well culture plates (Nunc, Inc., International, Roskilde, Denmark, 1 mL/well) both with and without quercetin (AppliChem GmbH, Darmstadt, Germany) at concentrations 0, 1, 10, and 100 ng/mL. These doses are comparable to the quercetin doses used in previous similar *in vitro* experiments with ovarian cells (Rice *et al.,* 2006; Jia *et al.,* 2011; Nna *et al.,* 2017). Medium alone was taken as control. Quercetin was dissolved in culture medium immediately before addition to the cells. After 2 d of culture with- and without quercetin, the culture medium from plate wells was aspirated and frozen at –18°C until enzyme immunoassay (EIA).

Immunoassay

Accumulation of the hormones P4, T, and E2 was determined in 25-100 µL aliquots of the incubation medium by EIA as described previously (Münster, 1989; Prakash *et al.,* 1987). All EIAs were validated for use in aliquots of the culture medium by RIAs described previously (Sirotkin *et al.,* 2017) and by serial sample dilutions. The assay sensitivity of P4 was 0.12 ng/mL and cross-reactivity of the antiserum to pregnenolone, androstenediol, testosterone, estradiol, and cortisol was less than 0.001%. Intra- and inter-assay coefficients of variation did not exceed 8 and 13%, respectively. The sensitivity of T was 10 pg/mL. The antiserum cross-reacted <96% with dihydrotestosterone, <3% with androstenedione, <0.01% with progesterone and estradiol, <0.02% with cortisol and <0.001% with corticosterone. Inter- and intra-assay coefficients of variation were 12.3 and 6.8%, respectively. Estradiol concentrations were evaluated with an assay sensitivity of 5 pg/mL. The cross-reactivity of the E_2 antiserum was <2% to estrone, ≤0.3% to estriol, ≤0.004% to T and ≤0.0001% to P_4 and cortisol. The inter- and intra-assay coefficients of variation did not exceed 16.6 and 11.7%, respectively.

Statistical analysis

The data shown are the means of values obtained in three separate experiments performed on distinct days with different groups of ovaries obtained from 5-7 animals. Each group of ovarian fragments in each experiment (used for testing of one quercetin concentration) was conducted using 6 wells with one fragment obtained from different animals per well (=18 fragments per group in all the performed experiments). Assays of hormones in the incubation medium were performed in duplicate. The blank control values were subtracted from the value determined in cell-conditioned medium to exclude any non-specific background (less than 14% of total values). Hormone secretion rates were calculated per mg follicular tissue/day. Significant differences between experiments and groups were evaluated

by Student's t-test using Sigma Plot 11.0 software (Systat Software, GmbH, Erkrath, Germany); $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cultured rabbit ovarian fragments released substantial amounts of P4, T and E2. High production of E2 and relatively low production of P4 by cultured ovarian fragments indicated their low luteinisation in culture, which is associated with increased P4 and decreased E2 production (Sirotkin, 2014). Fragments cultured with quercetin at doses of 10 ($P<0.01$) or 100 ng/mL ($P<0.05$) released more P4 than tissues cultured without quercetin (quercetin at dose 0 ng/mL) (Figure 1A). Furthermore, addition of quercetin at dose 1 ng/mL increased the release of both T ($P<0.01$) (Figure 1B) and E2 ($P<0.01$) (Figure 1C).

The current study is the first that demonstrates how quercetin directly affects rabbit ovarian secretions and that it can directly up-regulate the release of all steroid hormones from the ovary. These hormones are considered as both markers and promoters of female reproductive functions (Sirotkin, 2014). Therefore, the stimulatory action of quercetin administration on rabbit fecundity *in vivo* reported previously (Naseer *et al.,* 2017) can be explained by its direct stimulatory effect on ovarian steroidogenesis.

Previous and present studies have demonstrated the qualitative differences of quercetin action on reproductive processes in different species. Quercetin suppressed ovarian steroidogenesis in pigs (Santini *et al.,* 2009) and humans (Whitehead *et al.,* 2002; Rice *et al.,* 2006; van Duursen, 2017), but promoted it in our experiments in rabbits.

Understanding the causes and biological significance of such species-specific differences in quercetin action on ovarian hormones requires additional studies. It is proposed that quercetin does not have its own receptors in the ovary, but can bind to ovarian oestrogen receptors, which can either up- and down-regulate enzymes regulating production and metabolism of steroid hormones (van Duursen, 2017). We cannot rule out that in some species the binding of quercetin to oestrogen receptors could activate, and in some species, can suppress these receptors or the post-receptor signalling pathways. It is also possible that ovarian cells of different species have a different number of oestrogen receptors which can mediate phytoestrogen quercetin effects (Sirotkin and Harrath, 2014). Furthermore, the existence of negative

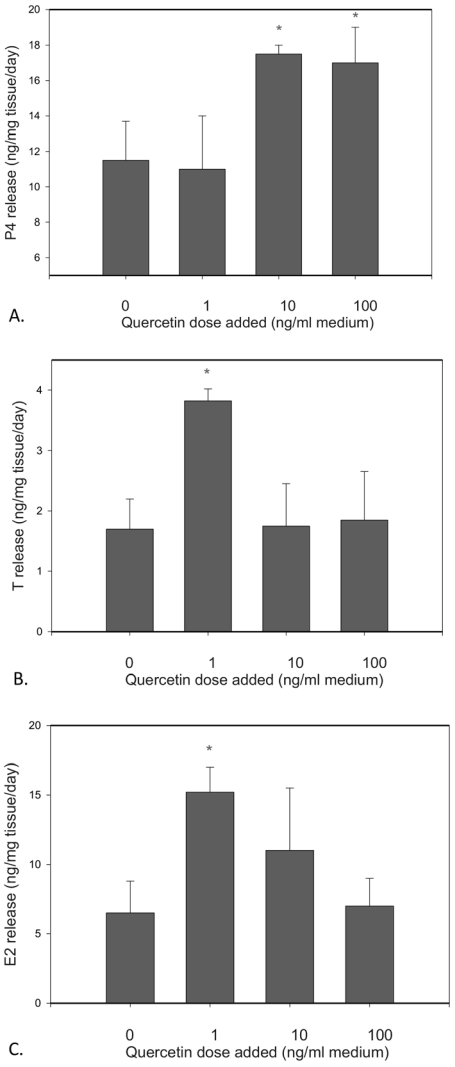

Figure 1: Effect of quercetin (0, 1, 10, and 100 ng/mL medium) on progesterone (P4) (1A), testosterone (T) (1B), and estradiol (E2) (1C) release by cultured rabbit ovarian fragments. Data of EIA. Each group was represented by totally 18 ovarian fragments collected from different animals and cultured separately. The values represent the means±standard error. *- indicates significant ($P<0.05$) effect of quercetin: differences between cells cultured with quercetin (1, 10, and 100 ng/mL medium) and control (quercetin at 0 ng/mL medium).

feedback mechanisms preventing the stimulatory action of quercetin at high doses in some species (but not in rabbits) could be hypothesised (see below).

Nor can it be excluded that consumption of a large amount of fresh food with quercetin in spring by prolific phytophage rabbit may affect its reproduction, as this animal can consume large amounts of this phytoestrogen, resulting in a positive influence on its reproductive system. The stimulatory action of dietary quercetin on rabbit ovarian functions and fecundity in summer has already been documented (Naseer *et al.,* 2017). In contrast, in less prolific mammals, for which reproduction does not depend on seasonal changes in quercetin consumption (for example, in humans), quercetin can decrease blood P4, T and E2 level via inhibition of steroid hormone synthesis and stimulation of their metabolisation (van Duursen, 2017).

It is also not to be excluded that the hyperactivation of ovarian steroid hormones receptors by phytoestrogen quercetin can induce negative feedback down-regulation of endogenous steroid hormones output. For example, in cultured porcine ovarian granulosa cells quercetin added at low dose promoted, but at high dose suppressed E2 release (Santini *et al.,* 2009).

The functional interrelationships between quercetin effects observed in our experiments require further clarification. P4 is a precursor of T, while T is aromatised into E2 (Sirotkin, 2014; van Duursen, 2017). Therefore, the increase in T and E2 release may occur because of an increase in the production of their precursors. For example, in non-ovarian cells, quercetin can either increase or decrease the activity of aromatase, enzyme converting T to E2 (van Duursen, 2017). Therefore, the promotion of T conversion to E2 in rabbit ovaries by quercetin is possible in principle. On the other hand, negative feedback mechanisms can prevent overproduction of some hormones by inhibiting their synthesis, which in turn could result in the accumulation of their precursors. Moreover, quercetin can affect rabbit ovarian steroidogenesis and reproduction via other, non-steroid regulators – hormones, growth factors and intracellular signalling molecules and metabolites, whose role has not yet been investigated (Sirotkin *et al.,* 2014). For example, quercetin influence on porcine ovarian cell steroidogenesis could be mediated by its action on Vascular Endothelial Growth Factor and redox status (Santini *et al.,* 2009).

The present and previous (Naseer *et al.,* 2017) studies demonstrated that quercetin can stimulate hormones and hormone-dependent ovarian functions, including fecundity in rabbits both *in vitro* and *in vivo*. These observations suggest that quercetin or quercetin-containing food could be considered as a potential nutritional bio stimulator of reproduction in rabbits, which could potentially be useful in rabbit production.

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Conflicts of interests: The authors declare that they have no conflicts of interests.

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REFERENCES

Anand D.A.V., Arulmoli R., Parasuraman S. 2016. Overviews of biological importance of quercetin: a bioactive flavonoid. *Pharmacogn. Rev., 10: 84-89. https://doi.org/10.4103/0973-7847.194044*

Beazley K.E., Nurminskaya M. 2016. Effects of dietary quercetin on female fertility in mice: implication of transglutaminase 2. *Reprod. Fertil. Dev., 28: 974-981. https://doi.org/10.1071/RD14155*

Boots A.W., Haenen G.R.M.M., Bast A. 2008. Health effect of quercetin: from antioxidant to nutraceutical. *Eur. J. Pharmacol., 585: 325-337. https://doi.org/10.1016/j.ejphar.2008.03.008*

Chen C., Zhou, J.J., 2010a. Quercetin: A potential drug to reverse multidrug resistance. *Life Sci., 87: 333-338. https://doi.org/10.1016/j.lfs.2010.07.004*

Chen Z.G., Luo L.L., Xu J.J., Zhuang X.L., Kong X.X., Fu Y.C., 2010b. Effects of plant polyphenols on ovarian follicular reserve in ageing rats. *Biochem. Cell. Biol., 88: 737-45. https://doi.org/10.1139/O10-012*

Münster E. 1989. Entwicklung von enzymimmunologischen Messverfahren auf Mikrotitrationsplatten zur Bestimmung von Testosteron und Progesteron im Blutplasma. *Doctoral Thesis. Institut for Animal Production and Breeding of the University of Hohemheim. 154.*

Naseer Z., Ahmad E., Epikmen E.T., Uçan U., Boyacioğlu M., İpek E., Akosy M. 2017. Quercetin supplemented diet improves follicular development, oocyte quality, and reduces ovarian apoptosis in rabbits during summer heat stress. *Theriogenology, 96: 136-141. https://doi.org/10.1016/j.theriogenology.2017.03.029*

Nna V.U., Usman U.Z., Ofutet E.O., Owu D.U. 2017. Quercetin exerts preventive, ameliorative and prophylactic effects on cadmium chloride - induced oxidative stress in the uterus and ovaries of female Wistar rats. *Food Chem. Toxicol., 102: 143-155. https://doi.org/10.1016/j.fct.2017.02.010*

Prakash B.S., Meyer H.H., Schallenberger E., van de Wiel D.F. 1987. Development of a sensitive enzyme immunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *J. Steroid Biochem. Mol. Biol., 28: 623-627. https://doi.org/10.1016/0022-4731(87)90389-X*

Rice S., Mason H.D., Whitehead S.A. 2006. Phytoestrogens and their low dose combinations inhibit mRNA expression and activity of aromatase in human granulosa-luteal cells. *J. Steroid Biochem. Mol. Biol., 101: 216-225. https://doi.org/10.1016/j.jsbmb.2006.06.021*

Santini S.E., Basini G., Bussolati S., Grasselli F. 2009. The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells *in vitro. J. Biomed. Biotechnol., 2009: 419891. https://doi.org/10.1155/2009/419891*

Sharma A., Kashyap D., Sak K., Tuli H.S., Sharma A.K. 2018. Therapeutic charm of quercetin and its derivatives: a review of research and patents. *Pharm. Pat. Anal., 7: 15-32. https://doi.org/10.4155/ppa-2017-0030*

Shu X., Hu X.J., Zhou S.Y., Xu C.L., Qiu Q.Q., Nie S.P., Xie M.Y. 2011. [Effect of quercetin exposure during the prepubertal period on ovarian development and reproductive endocrinology of mice]. *Yao Xue Xue Bao, 46: 1051-1057.*

Sirotkin A.V. 2014. Regulators of ovarian functions. *New York: Nova Science Publishers Inc. 194, ISBN 978-1-62948-574-4.*

Sirotkin A.V., Harrath A.H. 2014. Phytoestrogens and their effects. *Eur J Pharmacol., 741: 230-236. https://doi.org/10.1016/j.ejphar.2014.07.057*

Sirotkin A.V., Chrenek P., Kolesarová A., Parillo F., Zerani M., Boiti C. 2014. Novel regulators of rabbit reproductive functions. *Anim. Reprod. Sci., 148: 188-196. https://doi.org/10.1016/j.anireprosci.2014.06.001*

Sirotkin A.V., Kadasi A., Stochmalova A., Balazi A., Földesiová M., Makovicky P., Chrenek P., Harrath A.H. 2017. Effect of turmeric on the viability, ovarian folliculogenesis, fecundity, ovarian hormones and response to luteinizing hormone of rabbits. *Animal. 26: 1-8. https://doi.org/10.1017/S175173111700235X*

van Duursen M.B.M. 2017. Modulation of estrogen synthesis and metabolism by phytoestrogens in vitro and the implications for women's health. *Toxicol Res (Camb)., 6: 772-794. https://doi.org/10.1039/C7TX00184C*

Walgren R.A., Lin J.T., Kinne R.K., Walle T. 2000. Cellular uptake of dietary flavonoid quercetin 4'-beta-glucoside by sodium-dependent glucose transporter SGLT1. *J. Pharmacol. Exp. Ther., 294: 837-843.*

Whitehead S.A., Lacey M. 2003. Phytoestrogens inhibit aromatase but not 17beta-hydroxysteroid dehydrogenase (HSD) type 1 in human granulosa-luteal cells: evidence for FSH induction of 17beta-HSD. *Hum. Reprod.,18: 487-494. https://doi.org/10.1093/humrep/deg125*